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The main efforts were concentrated on the effect of pressure on the binding of nucleotides and potassium ions to purified (Na,K)-ATPase from pig and dog kidney, and on the equilibria between conformational states of the dephosphorylated enzyme. Trinitrophenyl-ATP was used as a fluorescent ATP analogue, and fluorescein enzyme conjugates were used as conformational probes. A modified high pressure bomb and spectroscopic cell for fluorescence measurements was designed and built. The results suggest that the potassium-occluded form of the enzyme is stabilized by pressure, presumably because it occupies a smaller volume, in agreement with predictions based on the effects of pressure on enzymatic activities.

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PRINCIPAL INVESTIGATOR: P. A. George Fortes

CONTRACTOR: University of California, San Diego

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INTRODUCTION

The aims of this project are to understand the effects of pressure on (Na,K)-ATPase at the molecular level. Our approach is to study the effects of pressure on the equilibria between different conformational states, subunit interactions, and partial reactions of this enzyme, monitored by site-specific fluorescent probes and chromogenic or fluorogenic substrates.

Because I am on sabbatical leave, the work during this first year of the project has been conducted in the laboratory of Professor Peter Jorgensen at the Institute of Physiology, Aarhus University in Denmark (25 July 1988 - 25 September 1988) and in the laboratories of Professor Gregorio Weber and Professor Enrico Gratton, at the Departments of Biochemistry and Physics, University of Illinois Urbana-Champaign (26 September 1988 to June, 1989).

The research work concentrated on studies of the effects of pressure on the binding of nucleotides and potassium ions to (Na,K)-ATPase and on the equilibria between conformational states of the dephosphorylated enzyme.

METHODS

Enzyme preparations. Canine renal (Na,K)-ATPase was purified (1) in my laboratory at UCSD by sucrose density gradient centrifugation in an angle rotor after treatment of the microsomal fraction from the outer kidney medullas with sodium dodecyl sulfate (SDS). Some of these preparations were labelled at cys452 and cys457 of the alpha subunit with 5-iodoacetamidofluorescein (IAF) (2), and others were labelled at lys501 with fluorescein isothiocyanate (FITC) (3). Porcine renal (Na,K)-ATPase was purified by isopycnic zonal centrifugation (4) in Aarhus. The latter method allowed separation of enzyme preparations that are pure and nearly 100% active, whereas the former method produces preparations that, although pure, contain a variable fraction 30-60% of inactive enzyme. All enzyme samples were frozen in liquid nitrogen and stored at -70 C. They were shipped to Urbana packed in dry ice.

Fluorescent probes. The functional enzyme concentrations were determined fluorometrically (5) with anthroylouabain, a specific probe of the ouabain site (6). Nucleotide binding was measured with trinitrophenyl-ATP (TNP-ATP), a nonhydrolyzable ATP analog that binds specifically with enhanced fluorescence to the ATP site of (Na,K)-ATPase (7). Conformational transitions were determined by TNP-ATP, IAF, and FITC fluorescence changes.

Instrumentation. My stay in Urbana has given me access to several commercial and home-built fluorometers and pressure equipment, which aided my selection of the instrumentation to be installed in my laboratory at UCSD. The fluorometers tested included the SLM 8000, the SLM 4800, the ISS Greg, the ISS K2, a Hitachi analog fluorometer, and a Princeton Instruments Optical Multichannel Analyzer (OMA), plus the phase-modulation lifetime instruments built in Professor Weber's laboratory and in the Laboratory of Fluorescence Dynamics headed by Prof. Gratton. Most of the work was done on the ISS Greg and in a home-built filter photon-counting instrument in Prof. Weber's laboratory. The OMA, although quite convenient because it can record full spectra in 30 milliseconds, could be used only in experiments with the fluorescein conjugates because of its low sensitivity and higher noise level.

The high pressure cell was the original Paladini-Weber design, which has quartz windows and a cylindrical sample container made of quartz and capped with polyethylene tubing to transmit the pressure. The pressure ranges studied were 1 bar to 2 kbar (the (Na,K)-ATPase is inactivated irreversibly above 2 kbar). The pressure was measured with a 16 inch diameter Heise gauge, which has markings every 5 bar.

RESULTS AND CONCLUSIONS

The experiments were designed to study the effects of pressure on the two main conformations of the dephosphorylated (Na,K)-ATPase: E_1 and $E_2(K)$. E_1 denotes the conformation that has high affinity for ATP, induced by incubation in the absence of ionic ligands of the enzyme or in the presence of sodium ions. $E_2(K)$ denotes the potassium-occluded form, induced by incubation with potassium ions, and has low affinity for ATP. The transition from the $E_2(K)$ form to the E_1 form is the rate-limiting step during normal turnover of (Na,K)-ATPase (8). Since turnover is inhibited by pressure, it is likely that the E_1 <---> $E_2(K)$ equilibrium is sensitive to pressure (9).

The fluorescent analogue of ATP, TNP-ATP, was used to study nucleotide binding to E_1 and $E_2(K)$. TNP-ATP binding to the ATP site of (Na,K)-ATPase increases its quantum yield about 11-fold (7). The affinity for TNP-ATP of the E_1 form is very high, with K_D = 30 nM. The $E_2(K)$ form has a much lower (about 100-fold) affinity for TNP-ATP (7,10). Therefore, the concentrations of enzyme and TNP-ATP were chosen such that equilibrium binding was poised at atmospheric pressure, and changes in TNP-ATP binding

were measured by changes in fluorescence as a function of pressure in the range 1 bar - 2 kbar at 20 - 23 C.

The important findings in these series of experiments were that TNP-ATP binding to the E_1 conformation of (Na,K)-ATPase, *i.e.*, in the absence of ionic ligands or in the presence of sodium ions (10 mM), was relatively insensitive to pressure, whereas TNP-ATP binding to the $E_2(K)$ conformation exhibited a higher pressure sensitivity and qualitatively different responses. In the E_1 conformation pressure decreased TNP-ATP binding, but almost no decrease was found in the first 500 bars, and measurable decreases required pressure in the range 1 kbar - 2 kbar. By contrast, in the $E_2(K)$ conformation (with 10 mM potassium ions) pressure increased TNP-ATP binding with significant increases observed already in the 1 bar - 500 bar range.

The above results allowed the design of experiments to determine the effects of pressure on the $E_1+K \leftrightarrow E_2(K)$ equilibrium. Incubation conditions were chosen such that a fraction of the enzyme would be in the E_1 and $E_2(K)$ forms by adding a low concentration of potassium (10 to 100 uM), and the TNP-ATP concentration was chosen such that the free TNP-ATP would be 2 to 3 times the dissociation constant for binding to the E_1 form, but well below that for binding to the $E_2(K)$ form. Then, if pressure favored $E_2(K)$ over E_1, an increase in pressure should decrease the fluorescence because E_1 would be converted to $E_2(K)$ which would be unable to bind TNP-ATP at these low concentrations. The results showed that inclusion of potassium ions at concentrations as low as 10 uM (the K_D for potassium is 75-100 uM) caused significant decreases in TNP-ATP fluorescence with pressures of 500 bar, and almost complete dissociation of TNP-ATP in the 1 kbar - 2 kbar range.

The following conclusions can be derived from the results. 1. Nucleotide binding to the E_1 form has a small, positive volume change. 2. Nucleotide binding to the $E_2(K)$ form appears to have a negative volume change. 3. The $E_2(K)$ form has a smaller volume than the E_1 form, even if formation of $E_2(K)$ involves loss of two charges per mole of enzyme, because it is known that binding of 2 K ions forms a neutral complex, and loss of each charge should have a positive volume change of about 20 ml. Thus, the volume change upon formation of $E_2(K)$ must be larger, and in the opposite direction of +40 ml/mole due to K binding to the enzyme. These results are consistent with the findings that pressure inhibits (Na,K)-ATPase activity if $E_2(K)$ is stabilized by pressure. It should be noted that the volume changes referred to above are system volumes, i.e., solvent + membranes + enzyme. The differences between conformations, however, must be attributed to the enzyme.

A significant problem in these experiments is accurate quantitation. TNP-ATP is a poor fluorophore, with quantum yield when bound to (Na,K)-ATPase < 0.01. The experimental design requires use of submicromolar concentrations to poise the system.

Therefore, the fluorescence signals are very weak, of the order of the intensity of the Raman peak or lower. In addition, scattered light by the membrane suspension contributes a significant blank signal with intensities, under optimized conditions, that are 25-100% of those of the fluorescence. Usually, blanks are measured in the absence of the fluorophore and subtracted from the measured signals. In the pressure cell, however, identical blank samples are not possible to measure because of differences in scattering and positioning of each sample bottle. Furthermore, I found that the light scattering increases with pressure. For these reasons, precise quantitation of the fluorescence changes in the above experiments and calculation of changes in TNP-ATP binding, dissociation constants, and volume changes are not yet possible.

I have spent a considerable amount of time attempting to reduce or eliminate the light scattering background in the pressure measurements. Despite the use of holographic gratings, stray light remained too high and required the use of a double monochromator or a single monochromator plus an interference or band-pass filter for the excitation, and a monochromator plus a cut-off filter in the emission. I have found that the remaining scattering is inelastic scattering, is partially depolarized, and is different from both Rayleigh and Raman scattering. The interesting finding is that this remaining scattering changes with both pressure and enzyme conformation, and thus, also contains information that may be useful, pending further study.

The above studies led to an improved design for the high pressure cell and the sample cell. The new pressure cell was custom built, has four windows, and a cylindrical stainless steel sample cell with flat windows and a piston for pressure delivery instead of a polyethylene cap. The improved design should minimize reflections, scattering and distorsions from the cylindrical quartz sample cell. The new cell has provisions for fiber optics, and sapphire windows that have a higher pressure resistance than that of quartz. The new cell was delivered last week, and initial testing showed that the sapphire windows, although cut at the optical axis, have much higher pressure-induced birrefringence that precludes their use in fluorescence polarization measurements. Therefore, two windows will be replaced with quartz to allow polarization studies.

On the basis of the instrument tests, I ordered the ISS K2 fluorometer, which has improved optics that result in improved excitation intensities and light collection efficiencies especially with the pressure cell, and is the only instrument in the market that can measure lifetimes and steady state spectra with photon-counting. The instrument will be installed in my laboratory upon my return to La Jolla, together with the custom-built pressure set-up which includes a 16 inch Heise gauge.

A complication of the experiments with TNP-ATP is the finding that the fluorescence signals from bound TNP-ATP are not entirely

stable, especially after pressure treatment and in the presence of potassium. The fluorescence decreases with a time course of minutes to hours. The reasons for this decrease are not clear, but they may reflect conformational drifts, as described for soluble oligomeric enzymes by Weber et al. This phenomenon will be studied further, and may provide important clues on the dynamics of membrane proteins. Experiments are underway at reduced temperatures (0 - 10 C) that slow down the fluorescence changes, so that signals are stable for several hours at atmospheric pressure.

Experiments, similar to those described above with TNP-ATP, were done with enzyme preparations labelled with IAF or FITC. These fluorescein conjugates respond to changes in enzyme conformation with changes in fluorescein fluorescence. These probes have a very high quantum yield, so that the scattering background is negligible, and the signals are quite intense. The results with both of these probes on the effects of pressure on the E_1 and $E_2(K)$ forms agree with the conclusions of the TNP-ATP experiments. The fluorescein probes however, seem to be reflecting also changes in pK of nearby groups, since the responses under pressure are pH-dependent in the pH 7-8 range (those of TNP-ATP are independent of pH). The pressure dependence of the fluorescein probes will be characterized further next year.

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